IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Jens PETERSEN
Title:	**POLYACRYLAMIDE HYDROGEL FOR THE TREATMENT OF INCONTINENCE AND VESICOURETAL REFLUX**
Appl. No.:	09/938,667
Filing Date:	08/27/2001
Examiner:	Blessing M. Fubara
Art Unit:	1618
Confirmation No.	2505

DECLARATION OF Dr. IEVA ANKORINA-STARK

UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Ieva Ankorina-Stark , hereby declare as follows:

(1) I am currently employed as Scientific Director of Contura International A/S. My qualifications are as detailed in an appended curriculum vitae (APPENDIX 1).

(2) I am not an inventor of the captioned application, and I have no personal interest in the disposition of the captioned application by the U.S. Patent and Trademark Office (PTO).

(3) I have reviewed and believe that I understand the captioned application and its pending claims.

(4) In relation to the application, I understand that a PTO action dated January 11, 2008 ("Office Action"), cites against the pending claims a Russian Patent No. 2,148,957 to Sknar. From my review of an English-language translation of the latter document (see

APPENDIX 2), I understand the Sknar patent to disclose administration of polyacrylamide hydrogel into the ostium ureteris submucosa of the ureter for the treatment of vesicoureteral reflux (VUR). A PTO action dated January 11, 2008 ("Office Action"), also cites against the pending claims US 5,798,096 to Pavlyk.

(5) I understand that the PTO relies on the Sknar and Pavlyk patent for the proposition that it was a known practice, when the captioned application was filed (*circa* August 2000, the "critical date"), to inject a polyacrylamide hydrogel into the ostium of the ureter to impede the flow of urine. The PTO also has taken the position, I understand, that a person knowledgeable in urology (A) would have been motivated, at the critical date, to inject acrylamide hydrogel into the urethra and (B) would have expected the hydrogel thus to act as a bulking material, increasing resistance to the flow of urine in the urethra, thereby to treat urinary incontinence (UI). Accordingly, I understand the PTO's position to be that the documented usage of a bulking agent such as polyacrylamide hydrogel to treat VUR would have suggested, at the critical date, using such bulking agent to treat UI.

(6) For the reasons elaborated below, however, I believe that, at the critical date, a person knowledgeable of bulking agents would not have been motivated to use polyacrylamide, or would not have reasonably expected success in using polyacrylamide, as a bulking agent to treat UI, even in view of the documented usage of polyacrylamide hydrogel as a bulking agent to treat VUR, as in the Sknar patent.

(7) I understand the PTO's position, as detailed in paragraph 5 above, to presume that the Sknar patent is indicative of general opinion in the medical field, at the critical date, concerning the use of bulking agents; namely, an opinion that that therapeutic use of polyacrylamide as a bulking agent was both safe and effective.

(8) To the contrary, however, the general opinion or bias on point was that polyacrylamide either was unsafe or ineffective as a bulking agent. Indeed, I am aware that a bias against the safety of polyacrylamide persists to the present day.

(9) For example, Kazachkov *et al.*, in *Arkh. Patol. 60*: 58-61 (1998) (English-language abstract provided in Appendix 3 hereto), reported in 1998 on a case of iatrogenic granulomatous pleurisy, which developed after cosmetic correction using a polyacrylamide hydrogel composition; De Bree *et al.*, in *Arch. Facial Plast. Surg. 6*: 204-06 (2004) (abstract in Appendix 3) also reported the development of severe granulmatous inflammation after injection of a polyacryamide hydrogel for cosmetic purposes; In 2003, Wang *et al.*, in *Zhonghua Zheng Xing Wai Ke Za Zhi 19*: 328-30 (2003) (English-language abstract in Appendix 3), advanced the viewpoint that the use of polyacrylamide causes pain, is ineffective, and can result in nodules and macrophagocyte infiltration. As recently as 2006, Xi *et al.*, in J. *Biomed. Mat. Res. Pt. A 78A*:283-290 (2006) (article in Appendix 3), reported problems with the level of residual acrylamide monomer content in the polymer samples that they had tested. Moreover, a number of contemporary review articles, such as Jordan *et al*, in *Journal of Materials Science: Materials in Medicine*, *15*: 519-522 (2004), evaluating the diversity of materials proposed as suitable bulking agents, omit any mention of polyacrylamides.

(10) As the publications mentioned in paragraph 9 evidence, it has only been relatively recently, and well after the critical date, that the medical field has begun to recognize that polyacrylamide is not necessarily unsuitable for use as a bulking agent. Previously, the bias in the medical field against the therapeutic use of polyacrylamide was based on the negative results of studies relating to the composition described in the Sknar patent. At the time, a prevalent viewpoint ascribed those negative results to the polyacrylamide material itself, which, in turn, engendered the pre-critical date negative bias against therapeutic uses of polyacrylamide.

(11) Confronting this bias and the negative impressions against the polyacrylamide hydrogel, Contura undertook to improve upon the latter and upon the subject technology in general. That is, Contura had recognized the need for improving the polyacrylamide hydrogels, if possible, and had begun research in the hope of producing a polyacrylamide material that was a safe and effective bulking agent.

(12) At its outset, the above-mentioned Contura research presumed that, by reducing the content of solid polymeric material in a polyacrylamide hydrogel, the documented immune response could be lessened or eliminated. Contura's initial endeavors were to challenge the bias, supported by the Pavlyk patent, that low solid-weight content polyacrylamide hydrogels were to fluid to be suitable as bulking agents. Contrary to expectation supported the Pavlyk patent, Contura developed a production process for preparing a hydrogel material that displayed adequate viscosity at a low solid-weight polymeric content

(13) Contura discovered that these low solid weight-content polyacrylamide hydrogels also were characterized by a high residual acrylamide monomer content. To address this finding, Contura refined the production process to include a washing procedure that reliably reduced the residual monomers, in the low solid weight-content hydrogels, to levels of less than 50 ppm, which was non-toxic in practice.

(14) Contura then applied the washing procedure to polyacrylamide hydrogels having higher solid weight-content, the equivalents of the polyacrylamide hydrogel compositions investigated in the Sknar patent. As a result, it was revealed that these equivalents also had high residual monomeric content, and that the washing procedure reduced that content to physiologically acceptable levels of less than 50 ppm.

(15) The discoveries mentioned in paragraphs 12, 13 and 14 engendered a realization at Contura that the negative performance of polyacrylamide hydrogels, as previously documented, was not a reflection of the performance of the material *per se*, but rather was the result-of an undesirably high residual acrylamide monomer content.

(16) Through its research, as I have recounted above, Contura came to recognize that polyacrylamide hydrogels with relatively high solid weight content were not innately toxic. This realization prompted Contura to discount solid weight content as a design criterion and instead to prepare polyacrylamide hydrogels, including ones with very high polymeric content, on the basis of their displaying suitably complex viscosities, which was desirable for purposes of a bulking agent. So doing, Contura contravened

the two prevalent schools of thought in the field, namely, that polyacrylamide hydrogels

(A) were toxic at polymeric contents of 3.6 to 9% and

(B) were not suitably viscous outside of that range.

Thus, Contura developed a polyacrylamide technology so as to allow for suitably viscous and safe materials at a much broader range of polymeric content, erasing the bias against polyacrylamide itself.

(17) Accordingly, it is my opinion that, at the critical date: a person knowledgeable in the field of bulking agents would have had a bias against the therapeutic use of polyacrylamide, due in part to negative results associated with the hydrogel compositions investigated in the Sknar and Pavlyk patents; and, as a consequence, the knowledgeable person would not have been motivated to use polyacrylamide as a bulking agent to treat UI, even in view of the documented usage of a polyacrylamide hydrogel as a bulking agent to treat VUR, as in the Sknar patent. By the same token, I believe that a person knowledgeable in the field of bulking agents, at the critical date, could not have reasonably expected success in using polyacrylamide as a bulking agent to treat UI.

(18) I further declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated:_____ By: _____

Ieva Ankorina-Stark, Ph.D.

APPENDIX 1

CURRICULUM VITAE

Name:	**Ankorina-Stark**, Ieva
Title:	Dipl. Biol., Dr. rer. nat. (Ph.D. equivalent)
Data of birth and place:	June, 7th, 1967, Riga, Latvia
Address (private):	Rødlersvej 2, 2990 Nivå
	☎ +45/49 14 98 76
	Mob: +45/20 73 51 11
	E-mail: ivas@contura.com
Family status:	Married, two children

PROFESSIONAL CAREER

Since May 2003	Scientific Director, Contura International A/S
Since May 2000	Medical Adviser, Contura International SA
April 1998 - April 2000	Assistant Professor at August Krogh Institute, University of Copenhagen
June 1994 - March 1998	Research Assistant at the Medical Policlinic, Experimental Nephrology, Münster, Germany, research undertaken for Ph.D.-Degree
Oct. 1993 - March 1994	Guest researcher at the Institute of Physiology, University of Göttingen, Germany
February 1993	Guest researcher at the Institute of Physiology, University of Århus, Denmark
1990 - 1994	Research Assistant at the Institute of Experimental and Clinical Medicine, Riga, Latvia
1985 - 1990	Secretary/Laboratory Assistant at the Latvian Academy of Sports
1984 - 1989	Coach in academic rowing

EDUCATION

December 1997	Earning of Dr. rer. nat. (Ph.D. equivalent) degree at the University of Münster (Germany)
June 1990	Diplome in Biology, Latvian State University, Riga, Latvia
Sept. 1984:	Begin of study of Biology in the Faculty of Biology at the University of Riga, Latvia
June 1984:	„Higher School Certificate"

PROFESSIONAL EXPERIENCE

- Leadership of R&D department
- Handling of all contact to external partners and key opinion leaders in the connection with product development
- Establishment and maintenance of the Product Development Design Control System in compliance with the ISO and FDA requirements
- Management of the new product development
- Planning, design and management of pre-clinical and clinical studies
- Preparation of IDE applications for class III medical devices
- Responsible for providing and preparing medical documentation
- Preparation of printed packaging material/Product labelling
- Education of the company staff and external partners

- Participation in international congresses, workshops, networking of key opinion leaders
- Literature monitoring and reviewing
- Experience with products and scientific background within plastic and aesthetic surgery, urology/gynecology, rheumatology/orthopedics and gastroenterology.
- Research expertise in physiology and cell biology (*in vitro* and *in vivo* models)
- Teaching of the University students, supervising of students taking one year research projects to attain degrees in Science
- Co-organization of qualifying exams for Sports Academy, Latvia
- Co-organization of international scientific meetings
- Administration of research grants

ADDITIONAL EDUCATION/COURSES

23. November 2000	One day course "Labeling", Danish Medical Devices Certification (DGM)
29. November 2000	One day course "Rules for Medical Device Approval in European Community" (DMDA)
15. March 2001	One day course "ISO 13485:200X New Device Standard", DGM
27. June 2001	One day seminar "The Update from the MDA and Recent Development in the Device Sector", Medical Device Agency
11.-12. October 2001	Two day course "Risk Management", Medicoindustrien
February-March 2003	Seven days course "Project Organization and Management", Magister Foreningen/Copenhagen Business School
25. November 2003	One day course "Clinical Trials", Medicoindustrien
6.-7. June 2005	Two day course "Design Control. Quality assurance in product development", Medicoindustrien
13.-14. September 2005	Two day course "Biocompatibility testing and management", Medicoindustrien
17. and 24. May 2006	PMA preparation, RAPS
23. January 2007	Getting manuscripts written and published, Clinical Device Group

PUBLICATIONS

15 publications (original articles), 2 thesis and 26 abstracts at national and international congresses

SCHOLARSHIPS

Carlsberg Foundation Research Scholarship (April 1998-March 2000)

LANGUAGES

Latvian - mother tongue, Russian – second language, English – fluently, German – fluently, Danish – fluently.

Ieva Ankorina-Stark

Nivå, 01.05.2007

APPENDIX 2

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RUSSIAN AGENCY
ON PATENTS AND TRADEMARKS

(12) SPECIFICATION OF INVENTION
TO THE PATENT OF THE RUSSIAN FEDERATION

METHOD FOR TREATING THE CASES FOR VESICOURETERAL REFLUX

ABSTRACT

FIELD: medicine. SUBSTANCE: method involves introducing polyacrylamide hydrogel into ostium ureteris submucosa of an ureter on the injured side by means of injection until infiltrate roll is formed. Before endoscopic operation being done, the urinary bladder is filled with physiological salt solution to its physiological volume. The urinary bladder is catheterized for 24-28 h after the injection. Reversed urine flow is eliminated due to pressure upon the ureter being increased. EFFECT: avoided recurrences and postoperative complications.

DESCRIPTION

The invention relates to medicine, and namely, to urology, and may be used for treating the cases of children's vesicoureteral reflux (VUR) of I – III degree.

Urinary reflux from the bladder into the ureter and renal pelvis is caused by insufficiency of the valvular mechanism of the vesicoureteral junction, which normally provides for urinary drainage only from the ureter into the bladder.

In accordance with the Classification of Heikel-Parkkulainen (1966), which had been taken as the basis of the International Classification of 1981 and is accepted in the world pediatric practice, 5 degrees of VUR are distinguished: In degree I urine is thrown up to the middle third of the ureter, in degree II – up to the renal pelvis, in degree III-IV – into the caliceal-pelvical system, in degree IV pyelectasia and coarsening of arches is noted, in degree V – deformation of the caliceal-pelvical system and hydronephrotic changes are observed.

During the recent years the specific weight of the urinary system pathology attains a growing importance in the morbidity structure of children because of severe complications, which accompany this kind of pathology. VUR is one of frequent forms of urodynamics disorders in children and one of the main causes of the urinary tract infection. It is revealed in

chronic pyelonephritis in 25-60% of cases and in children with VUR pyelonephritis is detected in 80-100% of cases. In 32-78% of patients with VUR the threat of kidney contraction against the background of chronic inflammatory process exists [Osipov I.B., Dzheliyev I.Sh. Pediatriya (Pediatry), 1994, No 6, pp.22-24]. Reflux nephropathy serves as a reason of 15-20% of all cases of terminal renal insufficiency in children and adolescents and as an important cause of hypertension in children. Thus treatment of children with VUR is of decisive importance, as untimely conducted treatment increases the risk of occurrence and complication of concomitant diseases. Thus, for example, in children who were inadequately treated, incidence of renal contraction was 17% and in children who were timely treated, it was only 4.5% [Winberg J. et al. Pediat. Clin. N. Amer. 1982, Vol.29, pp.801-814].

In the opinion of a majority of authors, in degree I-II VUR conducting medicamental therapy with antibacterial drugs {A Textbook of Pediatry, 1993] in combination with procedures (EHF, UHF currents, electrophoresis, phytotherapy) is sufficient [Osipov I.B., Dzheliyev I.Sh. Pediatriya (Pediatry), 1994, No 6, pp.22-24]. The generally accepted regimens of pyelonephritis therapy are usually used. Patients with chronic cystitis are additionally given local treatment (instillations with dogrose and sea-buckthorn berries oil, camomile decoction, 1% collargol solution), EHF, UHF, electrophoresis with furagin, furadonin), phytotherapy (medicinal tea, bog berry jam, dogrose, oat, parsley decoctions). In hyperreflectory bladder anticholinergic drugs (atropine and the group thereof), coenzymes and vitamins are used. Applying ultrasound and diadynamic currents to the region of the bladder and ureters, paraffin applications, ozocerite are rather efficient. In hyporeflectory bladder M-cholinomimetics or antocholinesterase drugs and hyperbaric oxygenation are used. In this case physician should be sure that further worsening renal function will not occur during this treatment. Otherwise, prolonged treatment of reflux can result in gradual death of renal function. Preventive therapy with antibiotics can be prolonged from 6 months to 2 years.

However, short courses of antibacterial therapy are efficient only in respect to microorganisms of low virulence, but recolonization with microorganisms with manifested virulence is possible; at the same time, treatment with antibiotics promotes the development of side effects (renal functional capability decreases). The instant kind of therapy causes a positive effect in 72.1% of patients with degree I-II VUR. In children with degree III VUR efficacy of antibacterial therapy is only 28% [Osipov I.B., Dzheliyev I.Sh. Pediatriya (Pediatry), 1994, No 6, pp.22-24].

Besides, a high risk of reflux nephropathy development is proved in medicamental therapy of children with a high degree vesicoureteral reflux [Shiryayev N.D. VUR in children: assessment of therapy results. Abstract of the Doctor of Medical Sciences degree thesis,

Moscow, 23 pages]. The following examination results in 6-8 months of treatment serve as an efficacy criterion of a complex medicamental therapy: normalization of urine, elimination of inflammatory process in the bladder, functional restoration of the sphincter apparatus of the ureter ostia, disappearance or decrease in reflux, stabilization of the pyelonephritic process. As is shown by the practice of leading urologists [Pugachev A.G. Urologiya I Pediatriya (Urology and Pediatry), 1989, No 1, pp.9-13], relapse of disease in medicamenal therapy is observed in 55% of cases.

In degree III VUR over 50% of children require surgical treatment, however, electrical stimulation of ureterovesical co-junction has a high efficacy (the Inventor's certificate No 1,558,424, 1990). Essence of the method consists in that electrical stimulation of ureterovesical co-junction is conducted in children on the diseased side with pulsed current of 4-5 Hz frequency in a pulse duration 8 ms, filling frequency 2.5 KHz and amplitude current values 2.5-25.0 mA, electrical stimulation being effected using an electrode-catheter, which is inserted into the ureter ostium for the length of the submucosal section of the ureter.

The instant method allows achieving a positive time course of VUR in 71.4% of cases complete disappearance being observed in 45.2% of cases. Duration (a course of 4-5 procedures every second day in unilateral VUR, 8-10 procedures every second day in bilateral reflux) and possibility of complications of frequent cystoscopy can be attributed to the drawbacks of the instant method.

Besides, if one fails to achieve positive results for a short term (1.5-2 months), then surgical correction needs to be performed as prolonged electrical stimulation can result in still greater renal dysfunction because of a reflux impact that continuously affects thereon [Pugachev A.G. Urologiya I Pediatriya (Urology and Pediatry), 1989, No 1, pp.9-13].

In all cases of degree IV and V reflux probability of spontaneous recovery is low and therefore, an early surgical treatment is conducted following prevention and confirmation of reflux progression.

Creation of a passive valve using a long narrow intravesical submucosal passage in the bladder is the main principle of surgical operations.

The Lich-Gregair's and Politano-Lidtbetter's techniques of extravesical and transvesical plasty are traditionally used.

The Lich's method was described in 1961: incision of the muscular layer is done on the side surface of the bladder without opening of the mucous membrane thereof. The incision length must correspond to a length of the dissected distal portion of the ureter. Thorough hemostasis is performed. A mobilized portion of the ureter is placed into the formed passage, which portion is covered by muscular walls of the passage and these walls are sutured with

catgut sutures. W. Gregair in 1964 had modified the Lich's method for performing anti-reflux operations in narrowing in the bladder-ureteral segment. In this case the ureter is dissected in the narrowing site and the bladder mucosa is opened. Then the margins of the dissected ureteral wall is sutured with the bladder mucosa. Further according to the Lich's method the ureter's mobilized portion is laid down and the passage is sutured.

The Politano-Lidtbetter's operation is described in the literature as early as in 1958 and it is successfully used till the present time. Essence of the method consists in that the ureter ostium is isolated by a circular incision together with intramural portion up to an external wall of the bladder. A catheter is preliminary inserted into the ureter, which catheter assists in isolation of this ureter segment. The length of the mobilized portion is 3-5 cm. The mucosa is opened 3 cm higher the ostium and through the defect thereof a clamp is brought through the muscular wall, the mobilized ureter is grasped and brought out into the bladder. Further a tunnel is created under the bladder mucosa up to the location site of this ureter's ostium, along which tunnel it is just brought through. Subsequently the ureter is sutured up by catgut sutures in the region of the old ostium. Defect of the muscular layer and submucosa is sutured [Lopatkin N.A. and Pugachev A.G. VUR, 1990, pp.68-74].

The essential drawback of this operation consists in that it is performed completely intravesically that can cause the development of a number of complications, since bringing the ureter through the peritoneal cavity or at a very acute angle into the bladder will subsequently lead to disorder of urodynamics. The number of relapses is up to 50%.

During the recent years, the Kohen's operation is usually considered to be the most promising one.. Essence of the method consists in that a catheter is inserted into the ureter ostium, is is secured with one interrupted suture to the bladder mucosa. A platform of 1.5-2 cm diameter is cut out around the ostium, intramural portion of the ureter (up to 3-5 cm) is cut out. A submucosal passage is bluntly created parallel to the interureteral plica in a transverse direction. The mobilized ureter is brought through this passage. Interrupted sutures are applied between the earlier dissected bladder mucosa together with the ostium and the mucous membrane of the newly created ostium located above the contralateral one. The region of the old ostium is sutured [Pugachev A.G. Urologiya I Pediatriya (Urology and Pediatry), 1989, No 1, pp.9-13].

Drawbacks of this method consist in that in a significant dilataion by using only an intravesical access it is impossible to model the ureter for a needed length and besides, some clinicians believe that following Khoen's operation a sclerozed distal segment of the ureter is preserved and retrograde catheterization of the bladder is significantly complicated. The number of relapses is up to 10%. This operation allows enhancing efficacy of surgical treatment of UVR up to 98% [Abramov K.S. Clinical-immunological efficacy of surgical correction of bilateral

VUR. Abstract of the Candidate of Sciences degree thesis. Moscow, 1990, p.19]. Generally a positive effect of anti-reflux operations was noted out in 92.9% of cases [Osipov I.B., Dzheliyev I.Sh. Pediatriya (Pediatry), 1994, No 6, pp.22-24].

Complications of the operations include:

1) pathological processes occurring at the site of operation: inflammation, necrosis of the ureter distal portion with formation of fistulas, stenoses of this portion etc.

2) early relapse of VUR on the side of operation, occurrence of a contralateral reflux;

3) inflammatory complications: cystitis, pyelonephritis;

4) general surgical complications: bleeding from the operation wound, purulence thereof.

The range of therapeutic measures in urology has significantly broadened along with appearance of modern endoscopes. Transurethral therapeutic endoscopy allows avoiding extensive operations, a prolonged epicystostolic drainage and promotes prevention of inflammatory diseases of the urinary tract.

The number of complication of transurethral surgery is significantly lower than in open surgical interventions, however, they exist in the form of secondary bleeding and different injuries of the bladder {The Textbook on Clinical Endoscopy, 1985, 189 pages].

However, progress in urological endoscopy achieved during the last century and appearance of novel instruments and methods for treating give evidence of the fact that this method is promising.

In addition to the operations listed above, reimplantation of the ureters can be used, which allows restoring patency of the upper urinary tract, while the problem of its positive effect on preservation of renal functions, development of the kidneys and preventing progression of chronic pathological process is discussible [Lemer G.R. et al. Pediat. Clin. N. Amer. 1987, V. 34. pp.747-770].

In recent years the injections of some substances into submucosa shell, such as a Teflon paste, an ox collagen [Kramer S.A.//Pediatric – 1990, - V. 85. - P. 872 – 878], have been used, as an alternative for the re-implantation method.

However, up till now, the question about the biological compatibility of these materials and their suitability for a prolong prevention of VUR is not clear.

In particular, it has been established that Teflon paste in implantation can cause inflammatory reactions and bovine collagen can cause allergic reactions that results in occurrence of postoperative complications [Beck Ch.L. Laringol/Rhinol. Otol. 1980, Bd 59, No 11, pp.715-718].

Besides, follow up of late results post implantation has revealed such a form of complication as medial and distal displacement of the implant that results in disorder of

urodynamics.

Recently short communications have appeared about using for the treatment of degree II-IV VUR a novel implant "Deflux", which consists of dextanomere particles in sodium gyaluronane, however the same drawbacks as those for the above listed implants are also inherent to it [Cappozza N. et al. The VIII European Congress of Urologists, Rome, 1997, p.9].

A method for treating vesicoureteral reflux using the Teflon paste "Polytech" [The VII European Congress of Urologists, 1986] is selected as a prototype of the instant invention.

The method consists in that the paste is administered at amount 0.3-1 ml by injection with a special syringe above the ureter ostium under the bladder mucosa at the level of 6 hours under visual control that allows elongating intramural portion of the ureter. Positive results were obtained in 85-90% of patients with congenital degree II VUR.

As was already indicated above, the drawback of this method is occurrence of postoperative complications and relapses; besides, efficacy of this method in degree III VUR is problematic.

The indicated drawbacks are eliminated in the claimed invention.

The task of the invention is the prevention of relapses and reduction of the number of complicating disorders in the treatment of children's vesicoureteral reflux of I – III degree.

The set task is resolved by that, before the endoscopic operation, the bladder is filled with a physiological solution up to the physiological volume, and after that the injection of polyacrylamide gel "Interfall" is made into ostium ureteris submucosa of an ureter on the injured side until the infiltrate roll is formed having the height of 0.7 – 0.8 of the ostium ureteris diameter, and after that an urethral catheter is installed into the bladder for 24 – 48 hours locating the orifice in the region of the ureter.

In the last decade the hydrophilic polyacrylamide gel (PAAG) "Interfall" has received wide clinic use. This gel is produced by Kiev factory of medicinal preparations on the bases of developments of Ukrainian chemists and medical persons. The advantage of this plastic material is the gel-like consistence permitting to introduce it soft tissues by the method of injection by a syringe and a needle [First International symposium. Development and Introduction of New Polymeric Implantants for Plastic Surgery. – Ukraine, - Kiev, - 1996 – 61 pages).

In comparison with the paste useful for endoprosthesis and comprised of Teflon particles dispersed in glycerol [Berghaus A. H.N.O. 1987, Bd.35, No 6, pp.227-233] or a aqueous solution of a highly purified degraded by polymerization degree bovine collagen useful for the same purpose [Ford Ch. Et al. Laryngoscope, 1984, No 94, pp.513-518], the proposed hydrogel provides for:

1. A more stable clinical effect that excludes the need in repeated endoprosthesis; this

effect is caused by that a crossed-linked polyacrylamide in any implant of a hydrogel is essentially one giant macromolecule. It is non-resorbable, it is not rejected and not fragmented, it is not subjected to destruction and besides, it well retains water as a dispersion medium.

2. Absence of pronounced aseptic reactions, which are observed in administration of the Teflon paste and absence of allergic reactions as those to the season bovine collagen.

Histological observations carried out on animals showed that the hydrogel PAAG "Interfall" causes moderately pronounced reactive events in surrounding tissues only during the first two weeks of follow-up.

An alternative change in the form of mucoid and fibroid swelling, hyanolysis, necrobiosis and necrosis is not noted, as well as foreign body granulomas with giant cells of foreign body resorption are not observed. Adaptation process of the tissues surrounding the gel is limited by organization processes in the form of formation of a gentle-fibered connective tissue capsule. These signs are characteristic of an inert foreign body and they give evidence of the fact that the gel PAAG "Interfall" is from the morphological point of view a low reactive material [The I International Symposium Development and Introduction of Novel Polymeric Implants for Plastic Surgery, Ukraine, Kiev, 1996, 61 pages].

In administration of the gel no signs of circulatory disorders in the form of vascular hyperemia or their emptiness, hemorrhages, swelling, pre-stasis, stasis, thrombosis, ischemia, infarction and embolism are revealed. All these properties of the gel PAAG "Interfall" found during experimental studies, allow significant lowering the number of postoperative complications in the use thereof. Besides, the instant hydrogel is not subjected to resorption, fragmentation and rejection. It is permanently present in the site of inserting thereof surrounded by a thin connective tissue capsule consisting of 1-2 rows of cells of fibrocyte type and connective tissue fibers that prevents expansion thereof along the inter-tissue fissures and getting into vascular lumens that excludes getting into blood and lymph of the gel particles and obstruction therewith blood and lymphatic vessels, that is confirmed by the absence of the gel particles in the regional lymph nodes, intertrabecular spaces as well as the lungs. These properties of the gel determine reliability and clinical efficacy in using thereof for the treatment of vesicoureteral reflux; in administering the PAAG "Interfall" into submucosal region of the ureteral ostia the implant acts as a special load to increase pressure on the ureter that results in elimination of reverse urine flow. Volume of the administered gel is selected individually in each particular case and it is established that the infiltration ridge formed following administration of the gel must be 0.7-0.8 of the ureter ostium diameter. Such ratio allows providing a free physiologic passage of urine from the ureter and preventing relapse of the disease.

The ureter ostium is a specific valve, wherein the front wall of the intramural portion that

is practically deprived of muscular fibers, in gradual elevation of intravesicular pressure is abated against the back muscular wall that prevents from urine regurgitation into the ureter. Because of this peculiarity, preliminary filling of the bladder with normal saline up to physiological level allows elevating accuracy of manipulations in transurethral endoscopic insertion of the gel and providing a visual control over efficacy thereof (preservation of urine passage from the ureter, absence of bleeding). Besides, in the process of filling the bladder elongation of the submucosal tunnel occurs that results in enhanced anti-reflux mechanism.

Postoperative urethral catheterization of the bladder for 24-48 hours with positioning the opening in the region of the ureteral ostium allows maintaining a low intravesicular pressure, releasing tension of the bladder during postoperative period and thus speeding up formation of a thin-wall connective tissue capsule around the inserted implant, that also serves as the prevention of the disease relapses.

Thus the essential distinctive features of the invention provide for achievement of the technical result.

Detailed description of the method and examples of a particular embodiment

The method is embodied in the following way. Transurethral endoscopic intervention is conducted on a urologic arm-chair with fixation of the lower extremities on supports under general anesthesia. Following treatment of external teguments with solutions of antiseptics, external genitalia are lined with sterile linen. All manipulations are performed with the Storz ureterocystoscope.

The endoscope is inserted into the bladder, which is preliminary filled with normal saline up to physiologic volume. The bladder mucosa, the ureter ostium and location thereof, submucosal section of the ureter are examined with subsequent selection of the implantation site.

Along the cystoscope channel an endoscopic needle is inserted (itd length from the catheter edge to the needle tip is 1 cm) with a guide wire and puncture is done into a preliminary selected site of the submucosa of the ureter ostium. The point located at a distance of 1 to 3 mm from the lower ostium edge is the most rational site of administration. Having been convinced that the puncture is correctly performed, the guide wire is withdrawn, the endoscopic needle is connected to the syringe filled with the polyacrylamide gel PAAG "Interfall", then it is mounted on a special device of the "gun" type and a small amount of the gel is extruded. If the needle is inserted correctly, then the hydrogel in administering forms an infiltration ridge in the submucosal layer of the ureter ostium. Administration of the gel is performed until the height of the infiltration ridge is 0.7-0.8 of the ureter ostium diameter, this typically requiring 0.3-1.0 ml of the gel. Chromocystoscopy by intravenous administration of 0.5 ml 0.4% indigo carmine solution is performed to elucidate urine passage.

Having been convinced that urine passage is preserved and there is no bleeding (in case of bleeding from the mucosa electrocoagulation is performed), the needle and the cystoscope are removed. An urethral catheter is inserted into the bladder locating the orifice in the region of the ureter ostium. A catheter of the Foley type having the size, which corresponds to a child's age (usually No 10-14 by the Sharier's scale) is positioned for 24-48 hours, and thereafter the catheter is removed. In bilateral reflux the same manipulation is performed in 1-6 weeks or simultaneously.

Patient is hospitalized for 3 days. A child is additionally prescribed a conventional antibacterial therapy for 1-3 months.

Disappearance or decrease in urination disorders, absence of relapses of inflammatory diseases serve as efficacy criteria of the conducted therapy.

The results are assessed using the following methods.

1. Monthly examination of urine seeding for 3 months post operation and then every 3 months.

2. Renal functional tests – determination of urea and creatinine, clearance of endogenous creatinine, the maximum urine osmolarity – once annually.

3. Excretory urography in 6-8 months post operation.

4. Miction cystography in 1-1.5 years post operation (only according to indications).

5. Ultrasonic or radioisotope examination in 6-8 months post operation.

Example 1. A child S-n, aged 8 years. Diagnosis: active degree III left vesicoureteral reflux, chronic pyelonephritis. The child was admitted to the Urological department of the Regional Hospital on 25 April 1997. Complaints of urinary incontinence at the day time at a peak of imperative urges. The number of urinations is 10-15; the efficient volume averages 40 ml. The urinary volume on the retrograde cystometry is 73 ml, the detrusor tone is 1.85, the sensitivity threshold is 31 ml.

Laboratory examinations: urinalysis of 25 April 1997: protein 0.033%, urea – 4.7 mmol/l, creatinine – 77 μmol/l, leukocytes – 8-10 x.

28 April 1997. Surgical treatment according to the claimed method has been conducted (the protocol No 343). The polyacrylamide gel PAAG "Interfall" was administered, an infiltrative ridge of the height making up 0.7 the ureter ostium diameter was formed. Postoperative period was uneventful.

29 April 1997 Ultrasonic examination of the urinary system.

Conclusion: the signs of VUR are not revealed.

30 April. The child was discharged from the Urological department with a complete elimination of the left VUR.

The child was followed up.

27 October 1997. A check up examination in 6 months.

The child has no complaints.

Ultrasound examination – the signs of VUR were not found.

Blood analysis: creatinine – 79 μmol/l, urea – 4.0 mmol/l.

Urinalysis: protein is absent, leukocytes – 0-0-1x, erythrocytes are absent.

Thus the operation conducted for degree III VUR developed using the PAAG "Inrefall" according to the above-described method, resulted in a complete recovery.

Example 2. A child I-v, aged 10 years and 11 months. He was admitted to the Urological department of the Regional Hospital on 15 September 1997. Diagnosis: bilateral degree III UVR, secondary chronic pyelonephritis, catarrhal cystitis. Complaints of nocturnal incontinence. Case history: therapy with uroseptics was conducted for 6 years, the signs of pyelonephritis and the bladder dysfunction periodically diminished but thereafter the disease recurred.

Laboratory examinations.

Urinalysis: protein – traces, leukocytes – 12-14x, red blood cells – 1-2 modified, epithelium – 10-15x.

Blood analysis: urea – 6.8 mmol/l, creatinine – 90 μmol/l.

16 September 1997. Ultrasonic examination: bilateral vesicoureteral reflux.

17 September 1997. Surgical treatment was conducted according to the claimed method (the protocol No 368). The PAAG "Inrefall" was bilaterally injected until a ridges having respectively the height making up 0.8 of the left ureter ostium and 0.75 of the diameter of the right ureter ostium were formed.

19 September 1997. Ultrasonic examination did not show the signs of VUR and urine drainage disorder for the both kidneys.

The patient was discharged from the hospital under follow up of the district urologists. A check-up examination was recommended in 3 months.

15 December 1997. A check-up examination.

The patient has no complaints.

Ultrasonic examination did not show the signs of VUR. Cystography did not reveal VUR.

Laboratory data.

Blood analysis: creatinine – 73 μmol/l., urea – 5.8 mmol/l,

Urinalysis: protein – absent, leukocytes – 0-0-1x, red blood cells – absent.

The given example supports obtaining a positive result in case of conducting an operation using the proposed method. Bilateral degree III UVR was completely eliminated that was supported by the results of ultrasonic examination and blood and urine analyses.

Example 3. A child M-ko, aged 1 year and 11 months. The child was admitted to the Urological department of the Regional Hospital on 10 September 1997.

Diagnosis: passive degree I vesicoureteral reflux, chronic pyelonephritis.

The patient complained of rare urination with small portions.

Case history: medicamenal therapy of pyelonephritis was conducted for 6 months.

Cystoscopy of 10 September 1997: Conclusion: non-closing right ureteral ostium.

Laboratory data.

Blood analysis: creatinine – 68 μmol/l., urea – 6.7 mmol/l,

Urinalysis: protein – 0.033 g/l,, leukocytes – 1-2x, red blood cells – 10-15x.

11 September 1997. Operation according to the claimed method was performed (the protocol No 360). The PAAG "Inrefall" was injected until a ridge the height making up 0.8 of the ureter ostium was formed. There were no complications of the operation. The child was discharged from the hospital under follow up of the district urologists. A check-up examination was recommended in 3 months.

1 December 1997. A check-up examination.

The patient has no complaints.

Ultrasonic examination did not show the signs of VUR. Cystography: the ureteral ostia are normal on the both left and right sides.

Laboratory data.

Blood analysis: creatinine – 88 μmol/l., urea – 5.3 mmol/l,

Urinalysis: protein – correspond to normal ranges.

The given example also supports possibility of a complete elimination of VUR using endoscopic prosthesis with the PAAG "Interfall". The obtained results of laboratory examination of blood and urine gave evidence of a complete recovery of the child.

Treatment of degree I-III vesicoureteral reflux according to the claimed technique was conducted on 167 children aged from 6 months to 15 years. Postoperative complications were not observed in any case. Follow-up of the patients for 1 year revealed disappearance of reflux in 92 children, decrease thereof down to degree I in 54 children, a relapse was detected in 14 children, in whom repeated injection of the implant was conducted, which resulted in disappearance of reflux and 7 children were operated according to the Kohen's method.

No macro- and microscopic signs of calcinosis in the sites of injection were found in administering the gel. In 6 months following administration of the gel the signs of cellular and tissue atypism were not detected. Beginning from three months a complete restoration of nervous fibers and their endings takes place.

No any signs of carcinogenic effect of the biogel PAAG "Interfall" on tissues, which

could be manifested by cellular atypism and cellular proliferation, were detected.

Parameters of urine and blood examination were within the normal limits. Seeding of urine did not reveal the presence of microbial flora; the parameters of retrograde cystometry were normal.

Thus the claimed method for the treatment of vesicoureteral reflux by administering as an implant the polycrylamide gel "Interfall" has a rather high clinical efficacy, it allows decreasing the number of relapses and complete excluding postoperative complications. Successful use of the claimed method for treating degree I VUR (in established practice of medicamental therapy) allows not only lowering medicamental load on the child body, but also preventing further progression of diseases. Contraindication to the use of the PAAG "Interfall" were not detected and in view of this, the method can be widely used in urologic practice.

CLAIMS

A method for treating of vesicoureteral reflux by injecting a biologically inert substance into ostium ureteris submucosa of an ureter, characterized in that the bladder is preliminary filled with a physiological solution up to the physiological volume, and the injection of polyacrylamide hydrogel "Interfall" is made on the injured side until the infiltrate roll is formed having the height of 0.7 – 0.8 of the ostium ureteris diameter, and, after that, an urethral catheter is installed, locating the orifice in the region of the ureter ostium, for 24 – 48 hours.

1: Zhonghua Zheng Xing Wai Ke Za Zhi. 2003 Sep;19(5):328-30.

Links

[Clinically analyzing the possible side-effects after injecting hydrophilic polyacrylamide gel as a soft-tissue filler]

[Article in Chinese]

Wang YB, Huang JJ, Qiao Q, Zhuang Q, Liu FH.

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OBJECTIVE: To evaluate the possible side-effects after injecting hydrophilic polyacrylamide gel for augmentation of the soft-tissue. METHODS: Fifteen patients with some side-effects after injecting the hydrophilic polyacrylamide gel had been undergoing for the treatment in our unit from 2000 to 2001. Their symptoms were analyzed and the specimen of the tissue was also removed for pathologic examination. RESULTS: The major complaints of the patients after injecting hydrophilic polyacrylamide gel were feeling pain(60.00%), uncomfortable (13.33%), no cosmetic improvement results (33.33%), secondary deformity (20.00%), long-lasting swelling (6.67%), and nodules (80.00%). The pathologic examination was showing the capsule formation (53.33%), macrophagocyte infiltration (60.00%) and granuloma producing (20.00%). CONCLUSION: Clinical application of the hydrophilic polyacrylamide gel may result in some serious side-effects. It should be cautious to the physicians who may apply the product for clinic use.

PMID: 15179867 [PubMed - indexed for MEDLINE]

APPENDIX 3

1: Arch Facial Plast Surg. 2004 May-Jun;6(3):204-6.

Severe granulomatous inflammatory response induced by injection of polyacrylamide gel into the facial tissue.

de Bree R, Middelweerd MJ, van der Waal I.

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Facial soft tissue augmentation has been performed using different materials. We describe a woman who received polyacrylamide gel injections for cosmetic reasons and developed a severe granulomatous inflammation paranasally. Because the patient did not mention the cosmetic intervention, the diagnosis of polyacrylamide gel-induced granuloma was complicated. The distinctive histopathological findings led to the correct diagnosis despite sparse clinical information. Since complete surgical excision was not feasible, she was treated with repeated multiple local injections of triamcinolone acetonide. Polyacrylamide gel may have favorable properties for facial tissue augmentation, but a severe granulomatous inflammatory response induced by injection of polyacrylamide gel may occur. Before treatment with polyacrylamide gel injection this complication should be disclosed to the patient.

PMID: 15148133 [PubMed - indexed for MEDLINE]

1: Arkh Patol. 1998 May-Jun;60(3):58-61.

Links

[Granulomatous pleurisy after mammaplasty, induced by polyacrylamide gel]

[Article in Russian]

Kazachkov EL, **Fridman AB**, **Friss SA**.

Chelyabinsk State Medical Academy.

A case of iatrogenic granulomatous pleurisy in a 36-year-old female developed after cosmetic correction of the mammary gland shape with polyacrylamide gel (an interfall injection). Symptoms of spontaneous left-side pneumothorax developed one month after the manipulation. Histological examination of the parietal and visceral pleura biopsy revealed multiple non-immune corpuscular mature macrophagal granulomas around gel particles resulting in the pleura damage and pneumothorax. A complete recovery. This is the first publication of pneumothorax of such genesis.

PMID: 9702306 [PubMed - indexed for MEDLINE]

Cytotoxicity and altered *c-myc* gene expression by medical polyacrylamide hydrogel

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Abstract: Medical Polyacrylamide Hydrogel (PAMG)has been used in plastic and aesthetic surgery for years. However, its safety is still in doubt in many countries. In the current research, first an approach, using high performance liquid chromatography (HPLC), to determine the amount of residual acrylamide monomer (AM) in the PAMG was presented. Then the cytotoxicity of PAMG was investigated using cell counting and methyl thiazolyl tetrazolium (MTT) assay. To explore the mechanism of this toxicity, normal human fibroblasts cultured in medium extracts were analyzed. Membrane changes and other related parameters were investigated using flow cytometry (FCM). Real time fluorescent polymerase chain reaction (real time PCR) was also introduced to determine the biological response of the fibroblasts. During this process, three representative genes (*p53*, β-*actin*, and *c-myc*, which are tumor suppressor genes, housekeeping genes, and proto-oncogenes respectively) were selected for examination. Results indicated that a method based on HPLC is practical and simple for determining AM in PAMG. The detection limits can reach the desired ppb level, and so it can fully meet the requirements of the studies of PAMG. Polyacylamide Hydrogel inhibits the growth of human fibroblasts and may cause the apoptosis of human fibroblasts. Moreover, it can alter physical parameters such as the size and the granularity of these cells. Furthermore, these three genes have a relatively typical amplification plot and highly related, wide-range standard curves, and so this reaction system is definitely suitable for the semiquantification of these genes. PAMG induces the increase of the message ribonucleic acid (mRNA) expression of *c-myc*, while the *p53* and β-*actin* remain even. This change is not related to the concentration of AM in the gel and may be incited by other components in the extract of PMAG. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 283–290, 2006

Key words: polyacrylamide Hydrogel; molecular biocompatibility; real time PCR; *p53*; *c-myc*

INTRODUCTION

Recently, medical Polyacylamide Hydrogel (PAMG), which is a watery gel consisting of approximately 2.5% crosslinked polyacrylamide and nonpyrogenic water, has been used for augmentation mammaplasty, but its safety is still in doubt in many countries.[1] In the product, there is inevitably residual acrylamide monomer (AM), which is said to have neurotoxicity,[2] genotoxity,[3] reproductive toxicity,[2] and potential carcinogenic effects.[4–8] Moreover, the combined effect of the remaining initiator, cross-linking agent, and AM is also worth exploring.

In this study, the first problem that needed to be resolved was the determination of the residual AM in the extract of PAMG. There are a lot of techniques that can be used to measure AM in fried food. They usually involve the use of gas chromatography with mass spectrometric detection (GC-MS) or liquid chromatography with tandem mass spectrometric detection (LC-MS).[9] For these methods, the preparation of the sample is complicated and tedious, and so they are not suited for the determination of AM in PAMG. Because

No benefit of any kind will be received either directly or indirectly by the authors.
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in the process of the quality control of the PAMG product and the study of its degradation in vitro or in vivo, repetitious manipulation for AM test is usually needed. Therefore it is very necessary for us to seek a more simple, accurate, and timesaving method for the quality control of the product and for the related studies.

Flow Cytometry (FCM) has become well-established in a number of biomedical and clinical areas.[10–12] The ability to determine multiple parameters of cell phenotype and function has consequently been utilized in a wide range of applications.[13–15] To explore the mechanism of the toxicity of PAMG, FCM was used to observe the effects of PAMG on human fibroblasts in detail.

The past three decades have witnessed a revolution in biomedical research, with the advent and rapid expansion of cell and molecular biology,[16] and the molecular biological approach has been applied to investigate the impact of biomaterials on cells.[17,18] Since message ribonucleic acid (mRNA) expression is the initial cellular response to a material, it is possible for us to know how cells recognize the material by studying what kind of mRNA is transcribed.[19] The most widely used method of evaluating mRNA expression of cells in contact with biomaterials is regular reverse transcription-polymerase chain reaction (RT-PCR) analysis.[20,21] With the advent of real time fluorescent polymerase chain reaction (real time PCR) tests, people tend to use this technique to quantify absolutely the gene expression of cells. It can even detect 10–100 copies of templates.[22,23] In real time PCR, the amount of the gene expression is judged by the number of the cycle in which the fluorescent value surpasses the threshold. By this way, it avoids the efficient difference of multiple in the late phase of amplification. As a result, it is more accurate than the regular RT-PCR. In biomaterial science, we usually need to acquire the relative level of gene expression rather than the absolute quantity. So this high-sensitivity method needs to be revised to become suitable for the quasi-quantification or the relative quantification of gene expression in the biomaterials research field.

The Gene *p53* is a tumor suppressor gene that encodes a nuclear protein that plays an essential role in the regulation of a cell cycle, specifically in the transition from G0 to G1.[24] It has been found that *p53* is transcribed and expressed at a very low level in the normal cell. In a variety of transformed cell lines, however, it is expressed in high amounts, and believed to contribute to transformation and malignancy.[25] The *c-myc* is a proto-oncogene that plays a key role in cell proliferation, differentiation, and apoptosis.[26] Overexpression of the *c-myc* gene has been implicated in the genesis of diverse human tumors.[27] So, in biomaterials science, both genes are often investigated to imply the carcinogenicity of biomaterials and to study the cellular response to biomaterials.[21]

In the current study, a practical method was established to determine the residual AM in PAMG. Then we examined whether or not PAMG affects the growth of human normal fibroblasts, which is one of the cell types that are typically found in the human tissue environment. To explore the mechanisms of these effects, FCM was carried out to measure the specific effects of PAMG on the cell membrane and on the physical parameters. Real time PCR was used to investigate preliminarily the change of expression of β-*actin*, *p53*, and *c-myc* in human fibroblasts, that are caused by the extracts of PAMG with different concentrations of AM.

MATERIALS AND METHODS

Materials

PAMG was obtained from Interfall Co. (Lot: 62000). Acrylamide, which is 99% pure and suitable for electrophoresis, was purchased from Sigma. Methanol (HPLC grade) was provided by Fisher Chemicals. Dulbecco Minimum Essential Medium (DMEM) was obtained from Hyclone. Annexin V-FITC Kit I was commercially available from BD Pharmingen. Both Trizol reagent and SuperScript One-Step RT-PCR with Platinum Taq were from Invitrogen. Sterile Natural Saline (NS) was supplied by Double-Crane Pharmaceutical. All of the PCR primers and Taqman probes are designed and synthesized by ABI. The probe was designed to span an exon/intron boundary to avoid genomic DNA amplification.

Sample preparation

PAMG was weighed and extracted by DMEM medium according to ISO 10993–12 guidance (1 g/5 mL of medium for 24 h at 37°C).[28] Then the extract was diluted with medium into different concentrations for the direct cell counting test. For other experiments, four groups of PAMG were weighed, dehydrated, and washed with methanol at 37°C for 24 h. Then they were dried to a constant weight at 37°C. These dried PAMGs were reconstituted to hydrogel by adding 20% initial quantity of water, and then sterilized at 0.105 MPa for 20 min. Finally, solutions with 5×10^{-6}, 5×10^{-5}, 5×10^{-4}, and 5×10^{-3} g/mL AM in sterile Natural Saline were added up to their original masses to form the experimental groups giving final AM concentrations for these groups of 4×10^{-6}, 4×10^{-5}, 4×10^{-4}, and 4×10^{-3} g/mL, respectively.

These experimental groups of PAMG were extracted similarly by sterile NS and by DMEM medium respectively according to the method described earlier. The sterile NS extracts were used to determine the concentration of AM in the extracts of PAMG, and the DMEM medium extracts

were applied to the related experiments on cells. The concentrations of AM in the later extracts were deduced by the counterparts of the former ones.

Determination of the concentration of residual AM in PAMG by HPLC

The high performance liquid chromatography (HPLC) system was an Agilent-1100 series consisting of G1311A quatpump, G1315B DAD, G1367A WPALS, and G1379A degasser. An ODS–3 column (4.6 × 250 mm, 5 μm particles; Inertsill, Japan) was involved in this method. The mobile phase was NS that had been adjusted to pH ~3.7 using hydrochloric acid, and the flow rate was 0.6 mL/min. Detection was performed by monitoring absorbance at 210 nm using a reference wavelength of 360 nm. The analysis was conducted at ambient temperature and the data were collected and analyzed on a data acquisition station using Agilent1100 ChemStation.

The stock standard solutions of AM were prepared by dissolving a known quantity of the monomer in 100 mL NS. A set of standard solutions was prepared by diluting aliquots of the stock solution with NS. These solutions were detected using this HPLC system and the standard curve for this system was set up.

To evaluate the recovery of this approach, the PAMG was precipitated by methanol, and then dried at 37°C for 24 h. The gel was reconstituted to hydrogel by adding a specific concentration AM solution into it. These samples were extracted by NS and analyzed by the earlier mentioned system.

For the determination of the concentration of the residual AM in PAMG, the sample was extracted by NS (1:5) for 24 h at 37°C. After being centrifuged at 3000 rpm for 5 min, the supernatant was filtered through 0.45-μm Syringe Filters before being injected into HPLC.

Cell culture and treatment

Normal human fibroblasts were obtained from the Medical Cell Center of the Chinese Academy of Medical Science. They were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone) at 37°C, in 95% humidity and 5% CO_2. The medium was changed every three days and for sub-culture. To detach the cells, the cell monolayer was washed twice with PBS and incubated with trypsin solution (0.25% trypsin). The detached cells were counted and seeded onto culture dishs with 2-mm grids (Corning Inc.). After 24 h, these dishes were washed with PBS and then treated with different concentrations of the extract of PAMG. The cells were counted under a microscope (Olympus) daily for 8 days.

For thiazolyl tetrazolium (MTT) assay, the detached cells were also seeded into 96-well plates. After 24 h, their media were replaced by the medium extracts of the experimental groups. The PAMG was replaced with an equal quantity of sterile NS to form the control. At 0, 1, 3, 5, and 7 days, cell proliferation was evaluated using the MTT assay, in which 0.01 mL of MTT (5mg/mL, Biomol) was added to each well and then incubated at 37°C for 4 h. At the end of the assay, the blue formazan reaction product was dissolved by adding 0.15 mL of DMSO to each well. The absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices SPECTRA Maxplus 384). The background absorbance produced by wells containing no cells was subtracted from all wells. The results are presented as the growth of each culture at each time period relative to the initial number of cells that is presented on day 0. This procedure thus accounts for possible differences in different samples.

Human fibroblasts were grown in polystyrene cell culture flasks (Costar) to approximately 75% confluence and then treated with corresponding DMEM extracts. After 36 h, the cells were released by trypsin, centrifuged, and collected for FCM analysis and for the determination of gene expression.

Flow cytometry

After being harvested by washing with PBS and incubating with trypsin solution as mentioned in the cell culture and treatment section, the cell concentration was adjusted to 1.0×10^6 cells/mL with 1× Binding Buffer. 100 μL of the solution was then transferred to a 5 mL culture tube. After adding 5 μL of Annexin V-FITC and 5 μL of PI, the cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Finally 400 μL of 1× Binding Buffer was added into each tube. These cells were analyzed by FCM within 1 h.

For FCM analysis, a FACScan flow cytometer (Becton Dickinson, Cowley, UK) equipped with a single Argonion laser was used. Minimums of 10,000 cells per sample were analyzed. Data were collected, stored, and analyzed with CELLQUEST software (Becton Dickinson).

Extraction of RNA

Total RNA was extracted using Trizol reagent (Invitrogen). Cells grown in monolayer, were lysed directly by adding 1 mL Trizol Reagent in each dish and the cell lysate was passed several times through a pipette. These homogenized samples were incubated for 5 minutes at 25°C to permit the complete dissociation of nucleoprotein complexes. A 0.2 ml drop of chloroform was added, and then these tubes were shaken vigorously by hand for 15 s and incubated at 25°C for 3 min. All of these samples were centrifuged at 12,000×*g* for 15 min. Following centrifugation, the aqueous phase was transferred to a fresh tube. After adding 0.5 mL isopropyl alcohol, these samples was incubated at 15–30°C for 10 min and centrifuged at 12,000×*g* for 10 min at 2–8°C. The RNA pellets were washed once with 75% ethanol and resuspended in diethypyrocarbonate-treated water. The concentration and purity of total RNA in each sample were determined by light absorbance at 260 nm and by calculating the A260/A280 ratio, respectively. Qualifying RNA samples should have a 260/280 ratio in the range of 1.8–2.0. All of these samples were diluted to 0.1 μg/μL to be ready for real time PCR analysis.

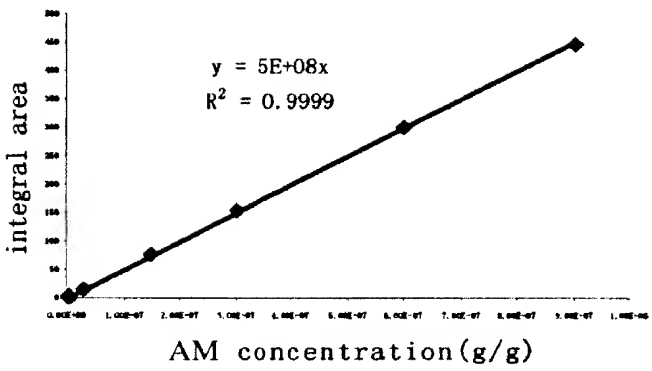

Figure 1. AM standard curve.

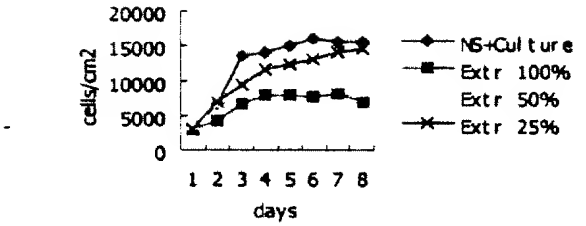

Figure 2. The growth curve of human fibroblasts in various concentrations of extracts of PAMG by cell direct counting.

Analysis of genes expression using reverse transcription real time quantitative PCR

Reverse transcription (RT) reaction and PCR were carried out for each RNA sample using the SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), according to manufacturer's protocol. Three genes were selected: β-*actin*, *p53*, and *c-myc*. Their RefSeq IDs in the National Center for Biotechnology Information (NCBI) are NM 001101, NM 000546, and NM 002467 respectively. Their assay IDs in ABI Corporation are Hs99999903 mL, Hs00153341 mL, and Hs00153408 mL. All other components except the total RNA sample were pooled in an Eppendorf tube, and then distributed into each PCR reaction tube. Finally, 10 μg total RNA was added. As a result, each reaction tube contained 50 μL PCR reaction mix, which contains 0.4 m*M* of each dNTP, 3.0 m*M* $MgSO_4$, RT platinum Tag Mix, Target Assay Mix, and 10 μL total RNA sample. RT-PCR reaction was carried out using an ABI PRISM 7000 Sequence Detection System (Applera Corp.). Cycling parameters were: 45°C for 15 min, and 95°C for 5 min followed by 45 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. Data points collected following primer extension were analyzed at the end of the thermal cycling. A threshold value was determined as 10 SD above the mean of the background fluorescence emission for all wells between cycle 1 and 15. The cycle number at which the fluorescence signal formed a positive sample crossing this threshold was recorded. Serial dilutions (10-fold) of a sample total RNA were analyzed for each target, and threshold cycle (Ct) was plotted versus the log of the initial amount of total RNA to give a standard curve.

Statistical analysis

To determine significant differences among the data, various statistical tests were utilized. In all tests, $p < 0.05$ was considered significant and null hypothesis was rejected. Specifically, paired *t* tests were performed to determine the significant effect of PAMG on the cell proliferation and physical parameters. The molecular effect of stimulation with the extract of PAMG was compared with the control by Univariate in SPSS 11.0. Values are expressed as mean ± standard error of the mean (M ± SD). Correlation analysis was performed using the Pearson's correlation analysis test.

RESULTS

Determination of AM level in PAMG by HPLC

This method provides good linearity with a wide range of AM concentration, which is from 0.003 to 0.9 ppm. The correlation coefficient (*R*) is 0.9999 (Fig. 1), and the recovery was more than 95%. The AM content in the extract of PAMG was 6.7×10^{-6} g/g in PAMG. Taking into account the dilution factor in the preparation of the extract, the AM content in PAMG should be 4.03×10^{-5} g/g.

Cell proliferation

Figure 2 shows the effect of different concentrations of PAMG extract on the growth of human fibroblast cells. It was found that different concentrations of PAMG extract had different effects on the proliferation of human fibroblasts. The degree of inhibition of cell proliferation increases as the concentration increases.

The results in Figure 3 showed that the cells in experimental groups proliferated over 7 days. The

Figure 3. Effects of the extracts of PAMG with different concentrations of AM on the growth of human fibroblast cells. *The result of extract with 10 e −3 AM cannot be obtained owing to the detaching of the cells from the bottonm of 96-well microplate in the MTT assay.

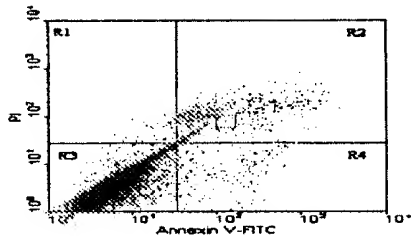

Figure 4. Reprensentative cytogram of annexin V binding (abscissa) vs. propidium iodide (PI) uptake (ordinate) in exponentially growing human fibroblast cells in culture with extract of PAMG at 36 h.

results are shown as the average in the number of viable cells on each day relative to the number presented initially on day 0, which is defined as 1.0. The vertical lines are the ±SD, obtained in three separate experiments. Statistical analysis of the data showed that the growth of the cells in the extract with 10 e −6 g/g AM was similar to the control and that the differences between them were not statistically significant. At day 5 and 7, however, the growth of the cells in the extracts with 10 e −5 g/g AM and 10 e −4 g/g AM were significantly lower than the control ($p < 0.05$).

Flow cytometry

Bicolor Annexin V/propidium iodide (PI) analysis revealed that the extracts of PAMG slightly induced the apoptosis of human fibroblasts (Fig. 4), which may be one of the reasons for the difference in cell proliferation. FCM analysis of the cells' size (represented by the forward light scatter (FSC)) and their granularity (represented by the side light scatter (SSC)) was also carried out to determine whether the material had any

TABLE I
FCM Analysis of the Effects of the Extracts of PAMG with Different Concentrations of AM on the Relative Size and Granularity of Human Fibroblast Cells at 36 h

Groups	Relative Size (FSC)	Relative Granularity (SSC)
10 e −6	91.6 ± 1.8	102.7 ± 6.9
10 e −5	84.5 ± 2.6	127.9 ± 2.9
10 e −4	102.0 ± 1.6	116.0 ± 9.1
10 e −3	92.2 ± 25.4	159.6 ± 16.8

effect on the physical characteristics of the cells. The representative dot plots shown in Fig. 5 indicated that human fibroblasts grown in the extracts of PAMG were smaller and more granular than the control. The results in Table I present a summary of 3 separate FCM analyses. The size and granularity of the cells are the average values (±SD) obtained in three separate experiments and are shown as a percent of the values obtained from the control cells grown in culture with the same portion of sterile NS. It shows that the average size (FSC ± SD) of the cells grown in the extract with 10 e −6 g/g AM was reduced slightly, whereas their granularity (SSC ± SD) was found to be similar to those of the control. The granularity of the cells in the extract with 10 e −4 g/g AM increased, although their sizes remained almost unchanged. For all the other extracts, including the 10 e −5 g/g and 10 e −3 g/g groups, the sizes were reduced and the granularities were found to have increased.

Reverse transcriptase real time quantitative PCR

The standard curves of these three genes were obtained by 10-fold serial dilution of a total RNA sample.

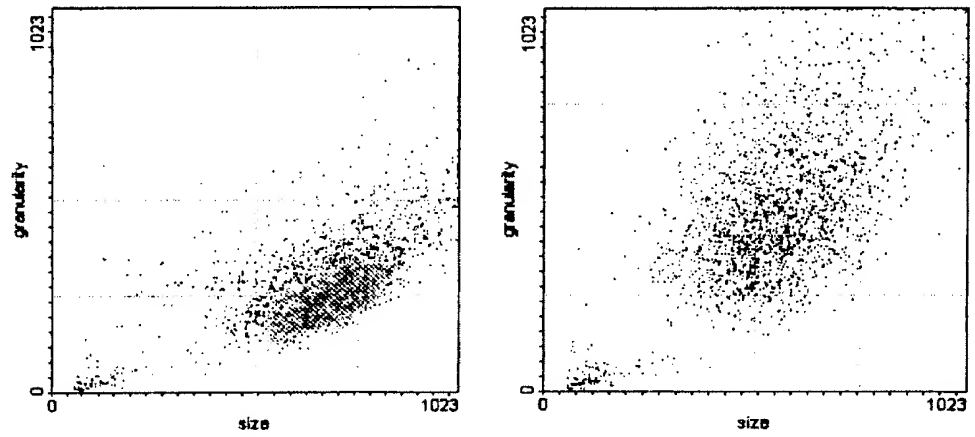

Figure 5. Representative FCM dot plot profiles of 2000 individual human fibroblast cells grown in the control (left) and in the extracts of PMAG (right) for 36 h, showing the size (FSC) and granularity (SSC) distribution of the cells.

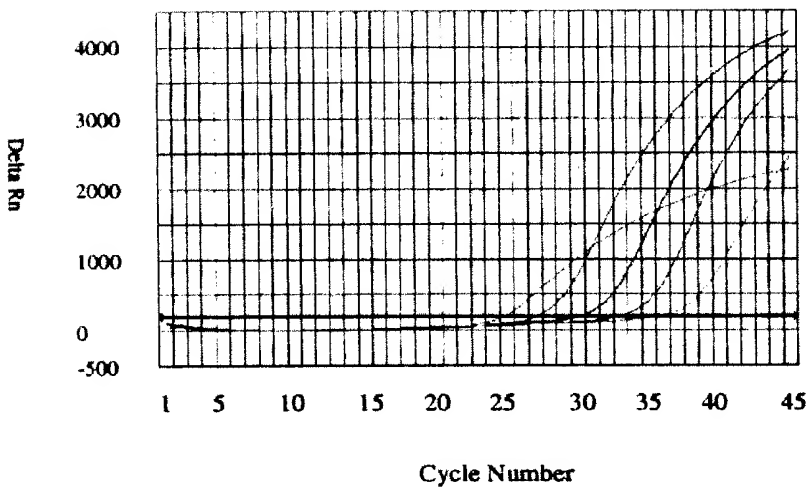

Figure 6. Typical amplification plot of e-myc in serial diluted samples.

Results showed that a relatively typical amplification plot of these genes can be obtained (Fig. 6) and that the correlation coefficients (R) of the targeted genes β-*actin*, *p53*, and *c-myc* were 0.999, 0.996, and 0.999 (Fig. 7) respectively. Results of the real time PCR showed that β-*actin* and *p53* mRNA expression levels in the experimental group and in the control were basically even, and that there were no significant differences between them ($p > 0.05$). For *c-myc* mRNA expressions, however, there were significant differences between these experimental groups and the control ($p < 0.05$). But there were no significant differences ($p > 0.05$) among these extracts with different concentrations of AM (Table II).

DISCUSSION

The aim of this work was to evaluate the effect of PAMG on normal human fibroblasts. The growth curves of cells in different concentrations of the extract of PAMG were plotted by direct counting. Then the impacts on the cell membrane and physical parameters were investigated. The biological response of fibroblasts to PAMG was also evaluated using real time PCR, which provides a novel technique for half-quantitative detection of mRNA expression in the biomaterials research field.

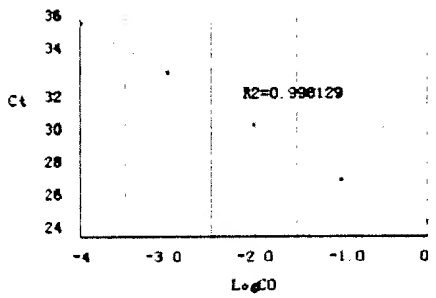

Figure 7. The standard curve of the mRNA of gene c-myc.

Here a simple method of determining AM in PAMG is presented. The detection limits of this system can reach the desired ppb level. It also has an excellent recovery. This method is based on the hypothesis that when the dried PAMG is hydrated with Natural Saline, the AM is uniformly distributed over the whole block of PAMG. The result of the recovery demonstrates that this hypothesis is definitely right. Overall, this method is simple, accurate, and timesaving, and so it is well suited for the control of AM in PAMG and for the repetitious manipulation during the study of its degradation *in vitro* or *in vivo*.

The results of this study showed that PAMG has obvious effects on human fibroblasts. From the cell growth curve, it can be seen that the extracts of PAMG inhibit the proliferation of cells, and the degree of this inhibition depends on the concentration of the extract. The result of HPLC showed that the residue AM in PAMG is at the level of 10^{-5} g/g. The residue AM is believed to be the major source of toxicity of PAMG, and so the effect of various dosages of AM on the proliferation of human fibroblasts should be observed. For this objective, AM was added into PAMG and then formed into the experimental group with different concentrations of AM. These PAMGs with altered AM level were extracted and evaluated by MTT assay. Results showed that the growth of fibroblasts is related to the AM level of the extract. Cell proliferation decreases as the AM concentration increases. So it can be concluded that the residual AM in PAMG can decrease the proliferation of normal human fibroblasts.

TABLE II
The Ct Values of the Three Genes' mRNA in the Control Samples

Mean	Control	10 e −6	10 e −5	10 e −4	10 e −3
p53	34.67 ± 1.98	33.41 ± 1.62	34.30 ± 1.66	34.58 ± 1.76	34.17 ± 1.80
β-*actin*	30.94 ± 1.95	30.95 ± 1.04	29.10 ± 1.46	31.48 ± 1.32	31.17 ± 1.88
*c-myc**	33.51 ± 1.55	29.29 ± 1.47	30.47 ± 1.19	30.82 ± 1.12	29.56 ± 1.73

In the early stages of apoptosis, changes occur at the cell surface. One change is the translocation of phosphatidyl serine (PS) from the inner side of the plasma membrane to the outer layer. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure to the cell membrane.[29] The present study showed that an extract of PAMG incited the exposure of PS, which is the early stage of apoptosis. The apoptosis and death of human fibroblasts incited by the extract of PAMG may be one of the reasons for the decrease in the number of the cells in these extracts. Furthermore, PMAG can also change the physical parameters of cells, such as their size and granularity. The result in Table I showed that different concentrations of AM have different impacts on the size of human fibroblasts, but they all tend to cause an increase in the granularity of the cells. Although the precise relevance of such changes to cell function is not clear, they are sometimes associated with the early stage of apoptosis in some types of cell.[30] This phenomenon needs to be further explored.

On the basis of these findings, it is necessary for us to investigate the molecular mechanisms underlying the inhibiting effect of these components on human fibroblasts. The mRNA expressions of a housekeeping gene β-*actin*[31], a proto-oncogene *c-myc*,[27,32], and the tumor gene *p53*[24,25] in human fibroblasts treated with extracts of PAMG were detected. The principle of reverse transcription real time PCR is to amplify the mRNA in cells to a detectable level so that the initial quantity of mRNA in cells can be determined by the threshold cycle (Ct) value. In this way, the expression of these genes in cells can be investigated. In the current study, the related coefficients of these three genes were all above 0.996, which showed that the reaction systems were stable and that they were suitable for relative quantification of the expression level of these genes in cells.

As we know, β-*actin* is a housekeeping gene, which tends to have a stable level in different tissues and different stages of cells. In this study, there was no significant difference in its expression levels between the experimental groups and the control, which reconfirms the stability of housekeeping genes and the reliability of the reaction system. The expression of *p53*, which is a suppressor gene in cells, remains even too. There were no significant differences not only between these extracts and the control but also among these extracts with different concentrations of AM. The patterns of *c-myc* gene expression, however, were different from the above two genes. Compared with the control, the *c-myc* gene was expressed more in cells that were treated with extracts of PAMG with different dosages of AM. But there were no significant differences between these samples that were treated with extracts having different levels of AM. So it can be concluded that the increase of *c-myc* mRNA expression is not related to the concentration of AM in extracts. Besides AM, there are also many other components in the extract of PAMG. Although it is noted that AM is the most important component that results in the difference of the proliferation of cells, the effect of other elements should not be ignored. This result is consistent with previous investigations.[33] The character and the quantity of the impact of other components in extracts should be further studied. The ultimate effect may be the synthesis of these two factors.

The *c-myc* gene has been implicated in many human cancers, and it is overexpressed in a subset of breast cancer.[34] Although the expression of *c-myc* is not sufficient for the generation of tumors, it may cooperate with other factors in the human tumorigenesis, or function as master transcriptional regulators of a wide array of target genes that execute the cellular response.[32,35] So, by evaluating the expression of these genes, the effect of PAMG on tumorigenesis could be assessed.

In biomaterial science, we usually just need to investigate the relative change of gene expression between different groups, and so the relative determination, rather than the absolute quantification, of the expression of selected genes is often used. In the current study, through the serial dilution of the sample, the standard curves of the destination genes were easily set up. By this way, the tedious and complicated process of cloning target genes in absolute quantification analysis was left out, which means that this method is perfectly suitable for application in the biomaterials field. Also, it overcomes the difference in the quality between the sample and the clone genes. Moreover, in comparison with the existing traditional RT-PCR, this method is simple and efficient. It has been shown that this approach is very feasible in this study. Therefore, this method is more convenient, accurate, and efficient than the regular RT-PCR, and it will have important applied significance in the inves-

tigation of the biological function of biomaterials at the molecular level.

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Novel injectable urethral bulking agents for the treatment of urinary incontinence

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Stress urinary incontinence is a highly prevalent disorder resulting from weak urethral closure mechanisms. Endoscopic injection of a urethral bulking agent (UBA) under the urethral mucosa increases coaptation, which improves continence. Collagen is an efficient agent, although its effects are limited in time. Other materials still suffer either from a short-lasting effect or migration in distant organs.

We evaluated here novel UBAs using an *ex vivo* model, with respect to criteria of ease of injection, ability to form a high and stable tissue bulking, implant elasticity and tissue reaction. One approach involves solutions of polymers in water-miscible organic solvents that precipitates *in situ*. In this manner, high and stable bulks were routinely obtained using various commercial polymers. Selected solvents reduced the tissue reaction to the implant. Microsphere suspensions in hydrogels also proved to be efficient UBA, although less stable bulks were obtained. Thermosetting chitosan hydrogels showed promising results with respect to bulk stability and isoelasticity with surrounding tissues. Different strategies have thus been compared and optimised *ex vivo*. Further experiments are required to compare the ability of these materials to induce a sustained *in vivo* bulking effect.

Introduction

Stress urinary incontinence results from a weak musculature of the bladder neck and urethral sphincter; it is a highly prevalent disorder in women but also concern men after prostatectomy. Endoscopic injection of an urethral bulking agent (UBA) under the urethral mucosa is an attractive minimally invasive procedure to treat stress urinary incontinence. The bulking agent produces a local tissue elevation which improves mucosal coaptation, hence urethral closure and continence. Collagen has gained acceptance as a safe and effective UBA, despite its short-lasting effect [1]. Various other materials have been proposed such as fat, particles suspensions made of poly(tetrafluoroethylene) [2], silicone [3] or carbon-coated zirconium [4]. However, these agents are not ideal due to short-lasting effect, particle migration [5–10], granuloma formation [5, 11], immunogenicity or volume loss [12]. The ideal material has yet to be developed [13].

We propose here different new strategies to produce urethral implants. These injectable products are: (i) charged latexes that coagulate in presence of physiological fluids, (ii) thermosetting hydrogels, (iii) microspheres suspended in a hydrogel carrier and (iv) solutions of preformed polymers in organic solvents that precipitate when in contact with water-containing tissues. Using an *ex vivo* model, we compared these new UBAs to collagen.

Material and methods

A commercial collagen-based UBA served as a control (Contingen, Bard, UK). The latex was a poly(vinyl acetate) (PVAc) aqueous suspension obtained by emulsion polymerisation of vinyl acetate, and subsequently purified by a two-day dialysis against water [14]. The highest latex concentration (20%) required the addition of 10% Lutrol F68 for stabilisation. The thermosetting hydrogel was based on chitosan and β-glycerophosphate, a compound liquid at room temperature that undergoes physical gelling when submitted to body temperature [15]. Poly(hydroxyethyl methacrylate) (PHEMA) or poly(hydroxyethyl methacrylate-*co*-methyl methacrylate) 20 : 80 (HEMA-co-MMA) microspheres were

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TABLE I Properties of selected urethral bulking agents. Bulk flattening is defined as the ratio of height after 4 h over initial height; n.m. stands for not measurable

UBA	Seringability	Backward flow	Bulking height (mm, mean ± std)	Young's modulus (kPa)	Bulk flattening (%)
Collagen control	2	3	5.8 ± 0.5	12.8	94
Latex PVAc 10%	3	3	4.3 ± 0.3		83
Latex PVAc 20%	3	2	5.0 ± 0	7.1	91
Thermoset chitosan 5% β-GP	2	2.5	7.0 ± 0.2	12.0	100
Thermoset chitosan 10% β-GP	2	1.8	5.5 ± 1.5	n.m.	100
HEMA-*co*-MMA 20 : 80 10% suspension	2.7	3	6.0 ± 0.5	n.m.	77
HEMA 10% suspension	2	2.5	3.8 ± 0.7	n.m.	57
EMA-*co*-MMA in DMSO	3	2	5.3 ± 0.4	21.5	103
EMA-*co*-MMA in NMP	3	2	5.0 ± 0.2	54.3	97
PMMA in GF 75	2	2	5.8 ± 0.3	28.8	96

produced by suspension polymerisation. HEMA was chosen for its biocompatibility with urethral tissue [16]. The microspheres were sieved to retain only those in the 80–120 μm range in order to avoid *in vivo* migration. The microspheres were suspended in different carrier gels or excipients: chitosan, sodium alginate, glycerol, hyaluronic acid or dextran 70.

As for the precipitating polymers, we used commercially available poly(methyl, ethyl or butyl methacrylate) (PMMA, PEMA or PBMA), copolymers of ethyl acrylate and methylmethacrylate (EMA-*co*-MMA), vinyl polymers (polyvinyl acetate) (PVAc), ethylene-*co*-vinyl alcohol (EVAL), and cellulose esters (cellulose acetate, CA, or cellulose acetate butyrate, CAB). The water-miscible organic vehicles have pharmaceutical or veterinary precedence. Dimethyl sulfoxide (DMSO) was used as a reference for its clinical precedence [17, 18], in addition to pharmaceutical excipients showing a reduced toxicity [19]: *n*-methyl pyrrolidone (NMP, ISP Technologies), dimethyl isosorbide (DMI, Uniquema), and Glycofurol 75 (GF75, gift from Roche, Basle, Switzerland).

The *ex vivo* model consisted of female porcine bladders with their urethra. We injected a constant volume (1.5 ml) through a 26-gauge endoscopic needle that is commonly used for submucosal peri-urethral injections. The bladders were immersed in saline at room temperature, or 37 °C for the thermosetting chitosan. In order to evaluate UBA efficiency, we measured the bulk dimensions up to 4 h after injection. We requested low bulk height variations (< 10%), and defined the bulk flattening as the ratio of the bulk height after 4 h over the initial height. We also required an easy injection and a limited backward flow of the polymer solution after needle withdrawal. The ease of injection or seringability was subjectively rated on a 3–0 scale (3: easily injectable, 2: injectable with little effort, 1: difficult to inject, 0: not injectable). Backward flow was rated similarly (3: no flow, 2: small tolerable flow, 1: important flow, 0: important flow leading to bulk flattening). The retrieved implants were examined by scanning electron microscopy, their elastic modulus was measured as the slope of the stress–strain curve for strains smaller than 10%. Following a 4-h incubation in an organ preservation solution (low-potassium dextran), selected formulations were submitted to histological examination.

Results

PVAc latex were easily injectable due to their low viscosity. Latex concentration of 10% did not lead to very stable bulks (83% of the initial height after 4 h, Table I) whereas 20% resulted in a more stable tissue elevation (91%). The bulks appeared as soft whitish mass (Fig. 1), with an elastic modulus of 7.1 kPa and dimensions comparable to collagen. However, the retrieved implants were also brittle, which would exclude them from clinical use.

Chitosan thermosetting gels were easily injectable and produced high bulks which were translucent (Fig. 1) due to the high water content. The β-glycerophosphate concentration impacted on the UBA performances, 5% being preferable for seringability and bulking effect (Table I). No bulk flattening was noticed. Elastic hydrogel implants were obtained (elastic modulus = 12 kPa).

To be injectable, the microspheres were suspended in a carrier viscous solution. We tested in preliminary

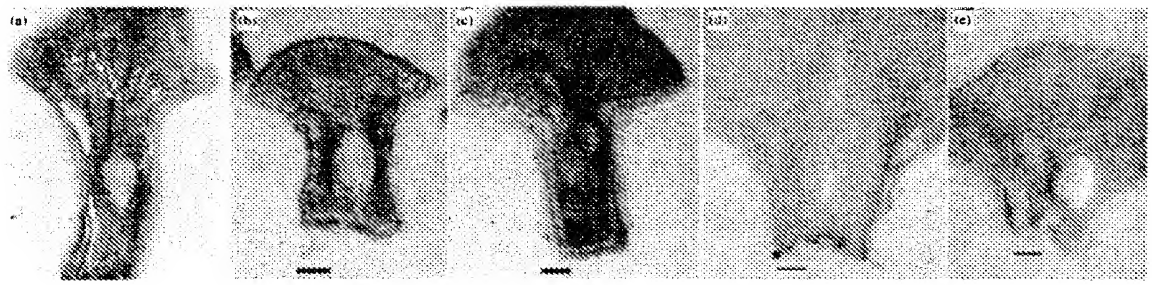

Figure 1 Macroscopic aspect of the urethras injected with (a) the collagen control, (b) a solution of EMA-*co*-MMA in NMP (c) a suspension of poly(HEMA-*co*-MMA) microspheres, (d) a thermosetting chitosan and (e) a PVAc latex. Scale bar = 1 cm.

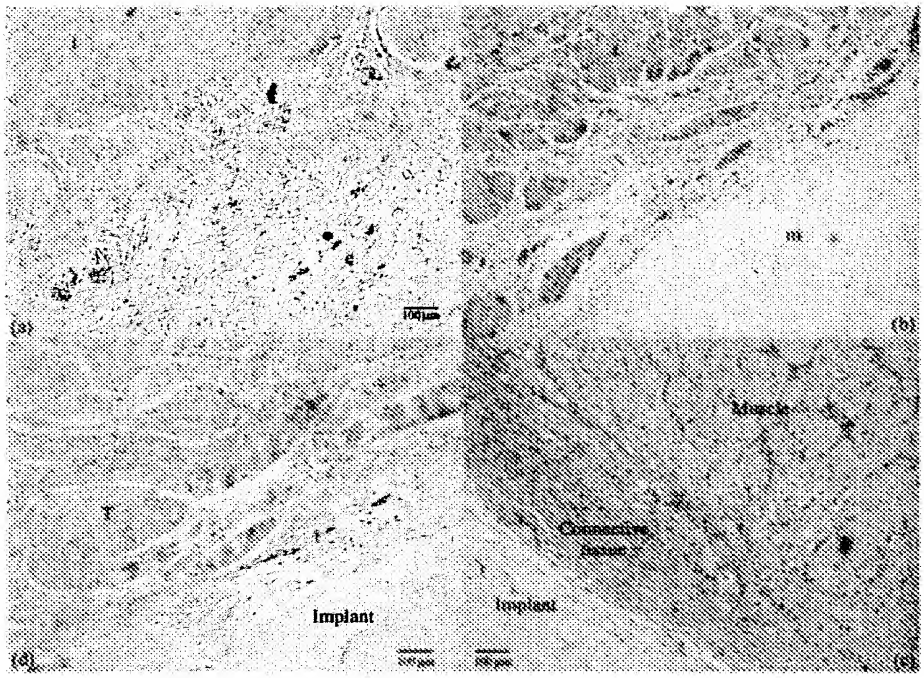

Figure 2 Histological sections stained with haematoxylin–eosin of: (a) a saline control, (b) a suspension of poly(HEMA-*co*-MMA) microspheres in dextran, precipitating polymers based on (c) cellulose acetate butyrate in DMSO and (d) EMA-*co*-MMA in NMP. Black arrows indicates a microsphere in (b). Scale bar = 100 μm.

experiments various solutions at different concentrations, based on sodium alginate, hyaluronic acid, chitosan, dextran 70 and glycerol. We selected an aqueous dextran solution with a concentration of 20% for its seringability, its ability to create high bulks over 4 h and to undergo autoclaving sterilisation without noticeable viscosity decrease. In addition, dextran is a well-documented pharmaceutical excipient devoid of adverse reactions. Suspension of either 10% microspheres made of HEMA-*co*-MMA (20 : 80) or made of pure PHEMA produced high tissue elevations comparable to collagen. Higher concentrations could not be injected through the 26-gauge endoscopic needle. Bulk flattening was noticeable (typically ranging from 60 to 80%) with these suspensions. No elasticity could be measured since the UBA remained viscous following injection.

The precipitating polymer solutions, once injected, produced microporous foams with pore size ranging from 10 to 100 μm. Various formulations resulted in efficient UBAs: PMMA in DMSO, DMI or GF 75; EMA-*co*-MMA copolymer in DMSO, NMP or GF 75 as well as EVAL and CAB in DMSO produced stable bulks comparable to the collagen control. A distinctive feature of the precipitating polymer solutions is the absence of bulk flattening after 4 h. The elastic modulus was slightly higher than with the aqueous-based solutions. It remained however in the 20–50 kPa range, still comparable to urethral tissue (13 kPa). In addition, we have shown that these implants did not loose their elastic properties after one year in saline (data not shown).

In order to assess possible tissue damage related to the presence of the organic vehicle, we carried out histological analysis of selected precipitating polymer solutions, using saline and microsphere suspensions as controls. Saline injection induced an oedema (e) that preserved normal subepithelial tissues (*t*, Fig. 2(a)), and suspension of poly(HEMA-*co*-MMA) in dextran did not either induce subepithelial tissue damage (Fig. 2(b)). Using DMSO-based solutions, some subepithelial tissue swelling was observed (Fig. 2(c)), whereas NMP, GF 75 and DMI seemed to preserve the tissues around the implants (Fig. 2(d)). Although thinner mucosae were generally observed around these implants, no mucosa rupture was seen.

Discussion

Despite significant advances in the field of urethral implants, current bulking agents still suffer from either a too short effect or migration of particles in distant organs. We evaluated here different new strategies for urethral bulking using a simple *ex vivo* model that allowed to select promising agents. The implants resulting form precipitating polymer solutions were generally stiffer than those obtained with aqueous-based implants (hydrogels, chitosan, latex, microsphere suspensions) and were shown to keep their elastic modulus over one year *in vitro*. In addition, they formed cohesive foams as opposed to the brittle material obtained with PVAc latexes. Permanent, biostable implants created by injection of precipitating polymers may therefore be good candidates to induce a sustained bulking effect. Our histological study indicated that pharmaceutical excipients such as DMI, GF 75 or NMP may be a valid alternative to the previously proposed DMSO [20] in order to decrease the short-term local reaction to the solvent. Similar implants based on NMP were shown to

be biocompatible when injected subcutaneously or intramuscularly [21].

Permanent implants may also induce a chronic, local reaction. Chronic granulomatous reaction of the giant cell type are known to contribute to the therapeutic success of implants for urinary incontinence [22], although this reaction may not be controlled on the long term and lead to implant failure [5, 11]. Biodegradable materials may therefore be of interest, more specifically if they induce and sustain the formation of new tissue. Chitosan is a biodegradable material of interest for bulking the urethra, since it is known for its wound healing properties and its stimulation of connective tissue formation [23]. Similarly, particle suspensions contain only a few percent of permanent materials that are expected to form new collagen and subsequent tissue bulking. The initial flattening of the bulk, that was attributed to the diffusion of the gel carrier, may be reduced *in vivo* due to local tissue reactions. The different strategies evaluated herein have been shown to induce short-term bulking effects on an *ex vivo* model. Their long-term fate *in vivo* has still to be confirmed in animal models.

Conclusion

Different strategies for urethral bulking were evaluated using a simple *ex vivo* approach. Solutions of preformed polymers in organic solvents resulted in permanent, porous implants. Thermosetting hydrogels and microsphere suspensions did also lead to efficient bulking, although their long-term fate *in vivo* will depend on their ability to induce a substantial long-lasting tissue proliferation. Further *in vivo* experiments will be dedicated to this issue. Novel formulations may induce a durable bulking effect, paving the way to clinically efficient agents.

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